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2. Patent application number *(The Patent Office will fill in this part)* — **9904251.7** — 24 FEB 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ZYLEPSIS LIMITED
6 HIGHPOINT
HENWOOD BUSINESS ESTATE
ASHFORD
KENT
TN24 8DH

Patents ADP number *(if you know it)* 6575575001

If the applicant is a corporate body, give the country/state of its incorporation — GB

4. Title of the invention — FLAVOUR/AROMA MATERIALS AND THEIR PREPARATION

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

YORK HOUSE
23 KINGSWAY
LONDON
WC2B 6HP

Patents ADP number *(if you know it)* — 109006

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month /year)*

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Request for substantive examination *(Patents Form 10/77)*	0
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11. I/We request the grant of a patent on the basis of this application.

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Date
23 February 1999

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Flavour/Aroma Materials and their Preparation

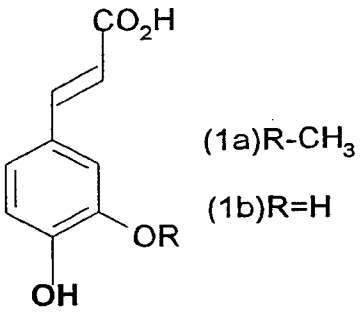
Background

The present invention relates to flavour/aroma materials and the preparation of such materials and key intermediates. It is particularly (though not exclusively) concerned with vanilla flavour materials and related materials.

Supplies of natural vanilla bean extracts suffer from shortages of supply and variability in quality. Despite a wide range of natural flavour chemicals having become commercially available over the last 10-15 years, no natural vanillin flavour chemical product or vanilla flavour has yet been developed. The main difficulties in developing a cost-effective vanillin product are firstly the unavailability of the preferred raw material, ferulic acid; and secondly the difficulty in finding microbial strains that can accumulate vanillin due to its ease of further metabolism, for instance to vanillic acid, and its inhibitory effect on the metabolism of cells. The main difficulty in developing a natural vanilla flavour is the large number of different vanillin-like molecules that together contribute to the superior flavour and aroma of vanilla bean extracts. In addition for the minor usage of vanilla in fragrances a colourless solid product is required rather than the coloured ethanol-

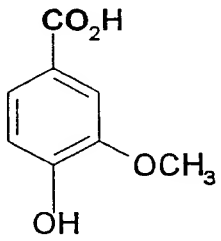
water vanilla bean extracts.

Our earlier application WO-A-96/39859 discloses the production of some phenolic materials by the enzymatic hydrolysis of plant materials. Thus ferulic acid (1a) was produced by enzyme treatment of wheat germ or wheat bran. Caffeic acid (1b) was produced by enzyme treatment of sunflower meal. Ferulic acid and esters thereof are valuable as precursor compounds and also as ingredients of foods and cosmetics, e.g. serving as antioxidants.

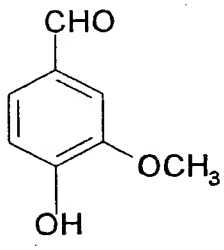


(1a)R-CH_3

(1b)R=H



(2)



(3)

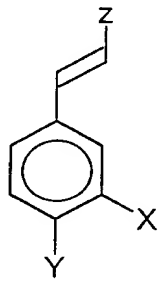
Various workers have reported the microbial conversion of ferulic acid (1a) into vanillin (3) either directly (e.g. DE-A-19532317) or via vanillic acid (2) (FR-A-2724394).

Outline of the Invention

Aspects of the present invention include the following.

A) A process for use in preparing a flavour/aroma composition containing a plurality of flavour/aroma components, said process having the step(s) of

(i) treating a plant material or plurality of plant materials to produce a precursor compound and preferably a mixture of precursor compounds preferably comprising two or more 1-phenylalkene species, preferably of formula (4):



(4)

where X and Y are independently selected from H, OH and OMe and Z is CO_2H or Me, most preferably comprising the compound (4) wherein X=OMe, Y=OH and Z=CO_2H (ferulic acid) and preferably also the compounds wherein X=H, Y=OH and Z=CO_2H (coumaric acid) and X=OH, Y=OH and Z=CO_2H (caffeic acid) ; and preferably

ii) subjecting said precursor compound or compounds (with or without separation from the plant material residues) to one or more biotransformations to produce a flavour/aroma composition. For precursor compounds having a benzene ring bearing a substituent -CH:CH-CO_2H (e.g. compounds of structure (4)), biotransformations may generate compounds in which this substituent has been converted into -CO_2H and/or -CH_2OH and/or -CHO.

Particularly preferred plant materials for step (i)

include maize, wheat, rice, sugar beet and parts thereof, particularly waste materials from their normal uses e.g. rice bran and cereal fibres. For example maize fibre and wheat fibre may be derived from dry or wet milling. Sugar beet fibre may be derived from pulp. A mixture of plant materials may be employed to give a desired mixture of precursors.

Step (i) may involve:

(a) treatment of plant material with acid (preferably citric acid, e.g. provided by addition of lemon or lime juice) to release glycosides of at least ferulic acid; and

(b) treatment of the glycoside-containing mixture with base (e.g. an alkali metal bicarbonate) or one or more enzymes with ferulic acid esterase ("FAE") activity to release ferulic acid. Suitable enzymes include Hemicellulase (from Amano, derived from Aspergillus spp.) and/or Celluzyme (Novo Nordisk) and/or enzyme from Humicola insolens, available as Biofeed Plus or Biofeed Beta (Novo Nordisk). Hemicellulase and Biofeed enzymes both have some xylanase activity, in addition to their FAE activity. If required, additional sources of xylanase can be added to supplement the zylanase activity already present.

In step (a), citric acid is suitable because it is reasonably strong, heat stable, cheap, active on a range

of ferulic acid containing materials, non-volatile, and not inclined to cause side reactions. It is adequately soluble in the cereal "mashes". It can easily be recovered as an insoluble salt (e.g. calcium), for reuse. It is a "natural" material, which is approved for food use. Alternatives include other organic polycarboxylic acids, particularly hydroxyacids, such as isocitric, tartaric, malic, fumaric and succinic acids.

Solutions containing suitable acids may be used, e.g. a grape-derived solution containing tartaric acid; or a fermentation medium containing citric or malic acid.

A microorganism may provide the activities required to carry out step (i)(b) and step (ii) (biotransformation of ferulic acid and/or other precursor compounds). For example we have developed strains of Aspergillus niger, A.flavus, and Penicillium chrysogenum having both the necessary FAE and alkene cleavage activities for acting on the product of the step (a) to convert ferulic acid glycoside into vanillic acid.

Step (i) can also be effected by treatment of plant material (e.g. maize fibre) with aqueous alkali such as a hydroxide, carbonate or bicarbonate of an alkali metal or alkaline earth metal. Sodium bicarbonate is preferred.

A product mixture containing vanillic acid and other materials (e.g. p-hydroxybenzoic acid from coumaric acid)

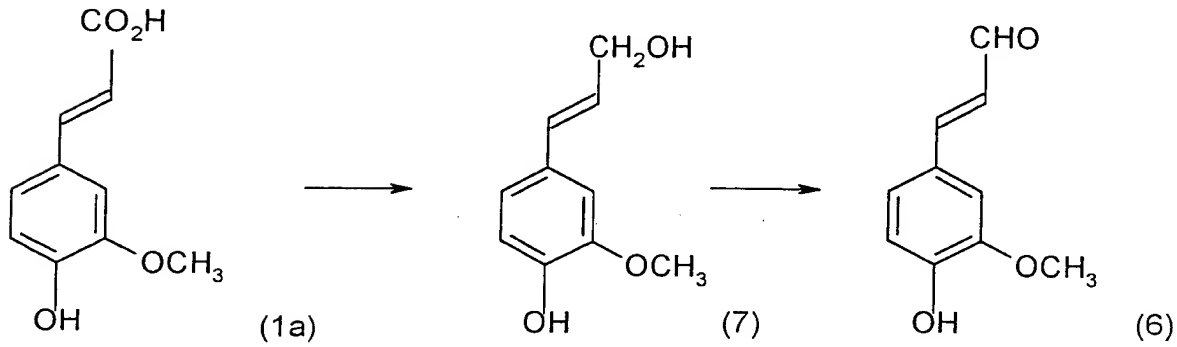
may undergo one or more further biotransformations without isolation of individual components, e.g. converting vanillic acid into vanillin and effecting corresponding transformations of other components.

B) Preparation of vanillin comprising the biotransformation of vanillic acid (2) into vanillyl alcohol (5), and the biotransformation of vanillyl alcohol (5) into vanillin (3). Generally the two steps will employ different microorganisms.

The steps may be novel in their own right. The reduction of vanillic acid to vanillyl alcohol may be effected by Zygorhynchus moelleri.

C) Preparation of vanillin comprising the biotransformation of vanillic acid (2) directly into vanillin (3) by means of strains of microorganisms such as Aspergillus fumigatus or Micromucor isabellinus.

D) Preparation of coniferyl aldehyde (6) comprising the biotransformation of ferulic acid (1a) into coniferyl alcohol (7), and the biotransformation of coniferyl alcohol (7) into coniferyl aldehyde (6), which may be converted into vanillin (3).



E) Preparation and isolation of substances, particularly substances prone to further reaction (e.g. aldehydes) or prone to inhibiting microorganisms (e.g. vanillin) by effecting a biotransformation using a microorganism in an aqueous phase which is brought into contact with a second phase into which products pass. This "in situ product removal" ("ISPR") may employ a vegetable oil as the second phase. Products may subsequently be recovered from the second phase, e.g. by crystallisation or solvent extraction. This process of ISPR can be employed in step (ii) of process A (above), or in processes B, C and D.

F) A vanilla flavour/aroma composition which is the product of a process according to (A) above or is a blend of one or more such products and/or one or more substances prepared by a process according to (B), (D), (E) or (F) above and/or one or more vanilla flavour chemicals from other sources and/or a vanilla bean

extract.

G) Preparation of ferulic acid (1a) by biotransformation of caffeic acid (1b), e.g. using Streptomyces griseus.

H) Use of Pseudomonas putida for converting ferulic acid into vanillic acid. The raw material is preferably the mixture obtained by treating plant material such as cereal fibre with citrate and FAE enzyme (as described in section A above), without isolation of the ferulic acid.

I) Various transformations as described above may be applied to other substrates. This may lead to further useful flavour/odour components. For example benzoic acid, 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acids can be converted into the corresponding aldehydes and/or benzyl alcohols. Protocatechuic acid (3,4-dihydroxybenzoic acid) can be converted into vanillic acid, analogously to the conversion of caffeic acid into ferulic acid.

J) A method of isolating strains of microorganisms for use in processes of the invention. Material (e.g. soil samples) containing a multiplicity of strains is used to produce a multiplicity of colonies, e.g. on agar, and individual colonies are tested for useful activity by means of a reagent suitable for detecting aldehydes. For example, 2,4-dinitro-phenylhydrazine produces orange/red

zones around aldehyde-producing colonies and a dark yellow zone around an alcohol producing colony.

K) Strains isolated by method J) and mutants thereof (obtained naturally, by conventional mutagenesis or by genetic engineering). This includes heterologous organisms which have been transformed so that they have derived activities of the "parent" organism from which the transforming nucleic acid was directly or indirectly derived.

Some particularly preferred strains have been deposited. Brief details follow.

a) Strains deposited with NCIMB

1. Brevundimonas vesicularis (Zyl 295)-NCIMB 40987-Gram negative bacterium

2. Pseudomonas putida (Zyl 503)-NCIMB 40988-Gram negative bacterium

b) Strains deposited with IMI

1. Rhodotorula glutinis (Zyl 702)-IMI 379894-Yeast, producing a red/orange pigment

2. Aspergillus flavus (Zyl 714)-IMI 379895-Filamentous fungus producing light green spores

3. Aspergillus fumigatus (Zyl 747)-IMI 379902-Filamentous fungus producing blue/grey spores

4. Trichoderma koningii (Zyl 751)-IMA 379903-Filamentous fungus producing green spores

5. Aspergillus niger (Zyl 759)-IMI 379904-Filamentous fungus producing brown/black spores

6. Rhizopus arrhizus (Zyl 779)-IMI 379898--Filamentous fungus, with aerial hyphae producing black spores.

7. Micromucor isabellinus (Zyl 849)-IMI 379893-Filamentous fungus producing pale brown spores

8. Zygorhynchus moelleri (Zyl 851)-IMI 379899-Filamentous fungus, with aerial hyphae producing black spores

9. Penicillium chrysogenum (Zyl 860)-IMI 379900-Filamentous fungus producing blue spores

10. Paecilomyces variotti (Zyl 733)-IMI 379901-filamentous fungus, yellow/brown spores

The use of those strains is illustrated in the following examples. Of course other strains of the same species may be used to carry out the same transformations.

The invention will now be explained in more detail, with reference to some specific examples.

General Experimental Conditions

In the following examples, where organisms are grown in culture broth, the growth medium can contain specified amounts of either, or both, of a vitamins supplement and a trace elements supplement.

These were prepared as follows.

Vitamins supplement: biotin (2mg L^{-1}), folic acid (2mg L^{-1}), pyridoxine (10mg L^{-1}), riboflavin (5mg L^{-1}), thiamine (5mg L^{-1}), nicotinic acid (5mg L^{-1}), pantothenic acid (5mg L^{-1}), vitamin B12 (0.1mg L^{-1}), 4-aminobenzoic acid (5mg L^{-1}), and thioacetic acid (5mg L^{-1}).

Trace elements supplement: concentrated hydrochloric acid (51.3 mL L^{-1}), MgO (10.75g L^{-1}), $CaCO_3$ (2.0g L^{-1}), $FeSO_4.7H_2O$ (4.5g L^{-1}), $ZnSO_4.7H_2O$ (1.44 g L^{-1}), $MnSO_4.4H_2O$ (1.12 g L^{-1}), $CuSO_45H_2O$ (0.25g L^{-1}), $CoSO_4.7H_2O$ (0.28g L^{-1}), and H_3BO_3 (0.06 g L^{-1}).

Analysis of coniferyl alcohol, coniferaldehyde, caffeic acid, coumaric acid, ferulic acid, vanillic acid, vanillyl alcohol and vanillin was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	Spherisorb C_{18}
Mobile phase	80:20 deionised water:acetonitrile containing 1% acetic acid
Flow rate	1.75 mL min^{-1}
Detection	Ultraviolet at 290nm

Analysis of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	Spherisorb C_{-18}

Mobile phase 80:20 deionised water:acetonitrile containing 1% acetic acid

Flow rate 2 mL min^{-1}

Detection Ultraviolet at 275nm

Alternatively, analysis of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde was carried out using thin layer chromatography (tlc) using the following conditions: silica plates eluting with petroleum either (40-60): ethyl acetate (50:50) and visualisation with UV or dinitrophenylhydrazine solution (0.4% in 2M HC1).

A) Use of Plant Materials

Maize fibre is an example of a cheap raw material which can provide raw materials for use in the preparation of flavour/aroma materials. Treatment with alkali leads to the liberation of ferulic acid, and lesser amounts of other materials, notably coumaric acid. The mixture can be subjected to biotransformation, e.g. to produce a mixture of vanillic acid and 4-hydroxybenzoic acid, which could be subjected to further biotransformations, e.g. converting the carboxylic acid groups to -CHO and/or $-CH_2OH$.

Examples 1A: Ferulic and coumaric acids from maize

(a) Use of sodium hydroxide: to 500g of ground maize fibre was added 1 litre of 1M sodium hydroxide solution and the resulting suspension was thoroughly mixed then

allowed to stand at ambient temperature (22°C) for 15 hours. Then 1 litre of ethyl acetate and 100mL of concentrated hydrochloric acid were added and the suspension mixed. The ethyl acetate phase was separated and the fibre suspension re-extracted with a further 1 litre of ethyl acetate. The combined organic solvent phases were dried (Na_2SO_4) and evaporated to dryness to yield a thick oil. Repeated washing of this oil with n-hexane gave a pale yellow solid comprising 33% ferulic acid and 2.9% coumaric acid.

(b) Use of sodium bicarbonate: to 10g maize fibre comprising 1.75%w/w ferulic acid (FA) and 0.1%w/w coumaric acid (CA) was added 100ml 0.5m sodium bicarbonate solution in a 250ml conical flask. The resulting suspension was heated and mixed at 85°C using a hot plate stirrer apparatus. The release of ferulic acid and coumaric acid into solution was monitored over time using hplc. Ferulic acid and coumaric acid yields were as follows:

60 min., FA 20mg, CA 1.6mg; 245 min., FA 100mg, CA 4.3mg; 315 min, FA 127mg, CA 5.6mg; 365 min., FA 126mg, CA 6.2mg. Yields at 365 min are equivalent to 72% and 62% release of available ferulic acid and coumaric acid respectively.

The maize suspension was coarse filtered by pressing

through a mesh bag, the recovered solids washed with 10ml deionised water and the filtrates combined prior to centrifugation (4,000 x g, 15 min). The pH of the supernatant was adjusted to pH 2.5 with concentrated hydrochloric acid followed by extraction with ethyl acetate (3x100ml). Evaporation of the combined ethyl acetate layers to dryness yielded 216mg of yellow/orange solid material comprising 41% ferulic acid and 2.7% coumaric acid.

In the following examples (c) and (d), the production of ferulic acid and coumaric acid from maize fibre was effected by a two step process. Firstly, acid hydrolysis of the fibre was achieved using citric acid solutions, supplied as either a defined quantity of citric acid dissolved in water or as the juice from freshly squeezed lemon or lime fruit. Secondly, the hydrolysis of solubilised cinnamate sugar esters was achieved by the addition of a hydrolytic enzyme preparation yielding ferulic and coumaric acids.

(c) Maize fibre (20g) was mixed with 100ml of citric acid solution (2%) in 250ml conical flask and heated at 126°C for 1 hour. The pH of the maize suspension was raised to pH 5.0 by the addition of 10M sodium hydroxide solution with vigorous mixing. An enzyme preparation (40mg, Hemicellulase, Amano) was added to the suspension

and the whole incubated at 50°C for 46.5 hours with mixing at 200rpm. The release of ferulic acid was monitored by hplc as described above. After 1.5 hours incubation a total of 51mg ferulic acid was present in solution, after 46.5 hours this amount had risen to 220 mg.

(d) Maize fibre (200g) was mixed with 1 litre citric acid solution (2%) in a 2L conical flask and heated at 126°C for 1 hour. The maize suspension was separated into insoluble solids and a liquor fraction using a wine press. First pressing yielded approximately 800ml liquor; the recovered solids were washed with a further 200ml of water and pressed again to give approximately 1 litre of combined liquor fractions. The pH of the maize liquor was raised to pH 7.0 by the addition of 10M sodium hydroxide with vigorous mixing. An enzyme preparation (Biofeed Plus L,2ml,Novo Nordisk) was added to the liquor and the whole incubated at 60°C for 7.5 hours with mixing at 160 rpm. The release of ferulic acid and coumaric acid was monitored by hplc as described above. After 7.5 hour incubation 1.6g L^{-1} ferulic acid and 0.1 gL^{-1} p-coumaric acid were detected in solution. Both cinnamic acids were recovered from aqueous solution by extraction into ethyl acetate as described below, followed by either, base extraction of the solvent to yield the cinnamate sodium salt, or evaporation of the solvent to dryness to yield

the cinnamate free acid.

Maize liquor (1L) was brought to pH 3 by the addition of concentrated hydrochloric acid and filtered through diatomaceous earth to remove insoluble material. The filtrate was extracted twice with 300 ml of ethyl acetate and the solvent extracts combined. Evaporation of solvent to dryness yielded 2.6g of orange solid comprising 1.39g ferulic acid and 0.1g coumaric acid, 53.5% and 3.8% purity respectively. In order to recover the cinnamates as their sodium salts, the ethyl acetate was continuously pumped over 10M sodium hydroxide solution (10ml) combined with vigorous mixing of the aqueous phase. This was continued until all but trace quantities of cinnamate had been recovered into the aqueous phase (approx. 2hrs). The aqueous phase was dried under vacuum at 45°C to yield 11.5 g of cream solid material comprising 1.3g ferulic acid and 0.1g coumaric acid, 11.3% and 0.87% purity respectively.

Example 1B: biotransformation of product mixtures

A seed stage culture of Rhodotorula glutinis (Zyl 702) was grown for 24 hours at 30°C with shaking at 200 rpm in a 250 mL shake flask containing 50 mL of minimal medium (containing 2g/l KH_2PO_4; 0.2g/l NaCl; 0.22g/l $MgSO_4$; 0.015g/l $CaCl_2$; 1ml/l trace elements solution; 10ml/l vitamins solution; 4g/l yeast extract; 4g/l

glucose). This culture was used to inoculate (2%) a 250 mL shake flask containing 50 mL of the same medium to which was added 300mg of the maize extract produced in example (a) above. This gave an equivalent to 2g L^{-1} ferulic acid and 0.18g L^{-1} coumaric acid substrates. The mixture was agitated at 500 rpm at 30° with a dissolved oxygen level of 60% of saturation. Substrate and product concentrations were measured by hplc as the following: 16 hours, ferulic acid 0.68g L^{-1}, vanillic acid 1g L^{-1}, coumaric acid 0.08g L^{-1}, 4-hydroxybenzoic acid 0.05g L^{-1}, 3,4-dihydroxybenzoic acid 0.02g L^{-1}; 18 hours, ferulic acid 0.14g L^{-1}, vanillic acid 1.54g L^{-1}, coumaric acid 0.035g L^{-1}, 4-hydroxybenzoic acid 0.08g L^{-1}, 3,4-dihydroxybenzoic acid 0.05g L^{-1}.

The product mixture can undergo further biotransformation, e.g. with reduction of $-CO_2H$ groups to -CHO and/or $-CH_2OH$ e.g. using Zygorhynchus moelleri or Micromucor isabellinus (see below). Thus the vanillic acid may be converted into a mixture of vanillin and vanillyl alcohol, in variable proportions. Likewise the 4-hydroxybenzoic acid may be converted into a mixture of 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol. The minor acid components may undergo corresponding reductions. Thus the end product is a complex mixture of odoriferous compounds, principally of vanilla type. The

proportion of aldehydes (such as vanillin) can be increased by biotransformation of the alcohols in the mixture, e.g. using Brevundimonas vesicularis.

Example 1C - Production of vanillic acid from Maize Fibre

Maize fibre (1kg) was subjected to citric acid hydrolysis (5L, 2% solution) followed by pressing and washing of the maize solids to give approximately 5 litres of maize liquor as described in (c) above. The pH of the liquor was adjusted to pH5.8 using 10M sodium hydroxide. A 2L aliquot of the above liquor was transferred into a 5L working volume fermenter and heated to 100°C for 1 minute. The remaining liquor was also heat-treated and stored separately. The fermenter was inoculated with 200 ml of a culture of *Penicillium chrysogenum* (Zyl 860) grown on pH5.8 maize liquor for 30 hours in a 1 litre conical flask (28°C shaking at 250 rpm). The fermenter contents were grown for 15 hours at 28°C while controlling the dissolved oxygen concentration at 30% of saturation. The pH of the culture was not controlled but remained unchanged throughout the incubation period. After 15 hours the volume of the fermenter contents was increased initially to 3.5L and then to 5L after a further 7 hours incubation. Incubation was continued as described previously for a further 3 days after which time the fermenter contents were assayed by hplc as described

above. The maize liquor was shown to contain 1.2gl^{-1} vanillic acid and only a trace quantity of ferulic acid. Fungal biomass was separated from the maize liquor by a single pressing of the fermenter contents through a wine press to give a clarified aqueous product.

Whereas P.chrysogenum (Zyl 860) is our currently preferred strain, Aspergillus flavus (Zyl 714) and A.niger (Zyl 759) are also usable.

Vanillic acid can be recovered from aqueous solution by extraction into ethyl acetate followed by either evaporation of the solvent to dryness or by precipitation of vanillic acid from the solvent by the addition of hexane. For example, a culture broth (900 ml) comprising 567 mg vanillic acid was acidified to pH 3.0 and extracted three times with ethyl acetate (1x400ml, 2x200ml). The solvent extracts were combined, assayed by hplc as described above and found to contain 485mg vanillic acid (86%recovery).

An aliquot (200ml) was taken from the combined ethyl acetate extracts and the volume reduced to approximately 10ml by evaporation. Hexane (40ml) was added slowly to this concentrated extract, accompanied by constant mixing, the precipitated material was recovered by filtration and dried to yield 140mg of pale yellow solid comprising 115mg vanillic acid (82% purity). Recovery

from the solvent was 88%, therefore, giving an overall recovery of 76% from the original culture broth.

Example 1D - Production of vanillin from maize fibre

The nutrient content of the clarified liquor from Example 1C was enhanced for growth of *Micromucor isabellinus* by the addition of nutrients as described in Example 4 below. No further vanillic acid was added to the liquor. A culture of *Micromucor isabellinus* (Zyl 849) was grown at 30°C for 3 days on pH 5.8 maize liquor (described in Example 1C) solidified with 1.5% agar. This culture was used to inoculate 20ml of the enhanced clarified liquor described above contained in a 100 ml conical flask. The flask contents were incubated at 30°C with shaking at 250 rpm for 24 hours, prior to being used to inoculate (2.5%) a second identical flask. Incubation conditions were as described previously. The concentrations of vanillic acid and vanillin in solution throughout the process were assayed by hplc as described above. After 24 hours incubation the pH of the liquor was slowly reduced over approximately 1.5 hours from pH5.2 to pH3.7. After 2.25 hours post onset of the pH reduction, $0.02gL^{-1}$ vanillin was detected in solution. After 4.5 hours the vanillin concentration had increased to $0.12gL^{-1}$. At 7.5 hours the vanillin and vanillic acid concentrations were $0.275gL^{-1}$ and $0.81gL^{-1}$ respectively. After 13 hours incubation the

vanillic acid concentration had decreased to 0.45gL^{-1} and the concentration of vanillin had reached a maximum at 0.35gL^{-1}.

B) Vanillin from vanillic acid via vanillyl alcohol

'One pot' bioconversions of vanillic acid to vanillin are known, but they generally show low yields and low conversion rates and/or low product concentrations (e.g. EP 453368, FR 2724394). We have found that Zygorhynchus moelleri can be used to produce very high concentrations of vanillyl alcohol (e.g. >5g/l) from vanillic acid. Vanillyl alcohol can be efficiently oxidised to vanillin, e.g. by means of Brevundimonas vesicularis.

Example 2: vanillyl alcohol from vanillic acid

A culture of Zygorhynchus moelleri (Zyl 851) grown on yeast malt agar was used to inoculate a 250mL starter culture flask containing 50mL medium, (20g glucose; 5g $(NH_4)_2SO_4$; 2g NaCl; $MgSO_4$ 0.22g/L; $CaCl_2$ 0.015g/L, 10mL; trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with pH6.0 phosphate buffer, 0.2M), containing 2g/L vanillic acid, which was incubated at 30°C with shaking at 200rpm for 24 hours. This starter culture was added to 5 litres of the same medium in a fermenter except that the medium components were dissolved in deionised water and also containing 2g/L

vanillic acid. This was stirred at pH 5.2, (30°C) for 24 hours after which the pH was altered to 3.5 over 1 hour, and the temperature kept at 30°C. Dissolved oxygen was maintained at 70% of saturation throughout the process. Assay was by hplc. Prior to the pH being allowed to drop (at the 24 hour stage), the amount of vanillic acid present in the system had not dropped and no products were seen. As the reaction proceeded, various amounts of substrate and nutrients were added as follows: 31.75 hours, 10g vanillic acid and 25g glucose; 47.5 hours, 50g glucose; 55 hours, 20g vanillic acid. Products were measured as being the following: 31.75 hours; vanillin, 0.26g/L, vanillyl alcohol, 0.3g/L; 47.5 hours, vanillic acid, 1.17g/L, vanillin, 0.1g/L, vanillyl alcohol, 2.38g/L; 55 hours, vanillic acid, 0.64g/L, vanillin, 0.06g/L, vanillyl alcohol, 3.5g/L; 5 days, vanillic acid, 1.21g/L, vanillin, 0.05g/L, vanillyl alcohol, 6.6g/L; after this stage no further product accumulation was observed. It is also evident that at pH 5.2 no substrate was converted into any products and the bioconversion only started when the pH was allowed to drop to pH 3.5.

Example 3: vanillin from vanillyl alcohol

Sterile nutrient broth (No 2) (50 mL) in a 250 mL shake flask was inoculated with Brevundimonas vesicularis (Zyl 295) and incubated at 30°C with shaking at 200rpm.

After 24 hours the addition of 50mg of vanillyl alcohol was followed by the flask being monitored regularly by hplc for both vanillyl alcohol and vanillin. Up to 71 hours post inoculation the amount of vanillyl alcohol decreased to around 10% of the original level and the amount of vanillin increased to approximately 90% molar conversion from substrate. Identification of the structure of the product was confirmed by nmr spectroscopy.

If the vanillyl alcohol was added at the time of inoculation of the flask then the amount of substrate did not start to decrease until after 16 hours incubation when vanillin was first detected. The overall conversions closely followed those seen when substrate was added after 24 hours.

C) <u>Vanillin from vanillic acid</u>

As mentioned above, the biotransformation of vanillic acid into vanillin is known, but the yields of known methods are poor, making them unattractive in commercial terms. FR-A-2724394 discloses a process, using a basidiomycete, which gives fairly good yields and conversion rates in percentage terms, but whose absolute yields are low. The highest yield of vanillin produced in an example is 628mg/l.

We have found strains capable of affording vanillin

at greater than 1g/l and usable in continuous production systems. Preferred microorganisms are strains of Micromucor isabellinus and Aspergillus fumigatus.

Example 4: production of vanillin by M.isabellinus

(a) A culture of Micromucor isabellinus (Zyl 849) grown on yeast malt agar was used to inoculate a 250 mL starter culture flask containing 50 mL medium (15g glucose; 5g $(NH_4)_2SO_4$; 2g K_2HPO_4; 0.2g NaCl; 0.2g $MgSO_4$; 0.015g $CaCl_2$, trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with deionised water) containing 2g/L vanillic acid which was incubated at 30°C with shaking at 200rpm for 16.5 hours. This starter culture was added to 5 litres of the same medium in a fermenter with vanillic acid added to a concentration of 1.5g L^{-1}. The fermenter contents were stirred at 30°C with the dissolved oxygen concentration being maintained at 70% of saturation throughout the process. Assay was by hplc as described above. As the reaction proceeded, additional amounts of vanillic acid were added as follows: 22.5 hours, 2.5g; 24 hours, 2.5g; 25.75 hours, 1g; 26.5 hours, 1g; 27.5 hours, 1g; 31 hours, 2.5g. Substrate and product concentrations were measured as being the following:: 22.5 hours, vanillic acid 0.6g L^{-1}; vanillin 0.84 g L^{-1}; 24

hours, vanillic acid .1.36gL^{-1};vanillin 0.96 g L^{-1}; 25.75 hours, vanillic acid 1.15g L^{-1} ;vanillin 1.1g L^{-1}; 26.5 hours, vanillic acid 1.27g L^{-1} ;vanillin 1.17 g L^{-1}; 27.5 hours, vanillic acid 1.43g L^{-1};vanillin 1.33 gL^{-1}; 31 hours, vanillic acid 1.16gL^{-I};vanillin 1.6 gL^{-1}; 46 5 hours, vanillic acid 1.58g L^{-1} ;vanillin 1.70 g L^{-1}.

(b) A culture of Micromucor isabellinus (Zyl 849) grown on yeast malt agar was used to inoculate a 500 mL starter culture flask containing 100 mL medium (15g glucose; 5g $(NH_4)_2SO_4$; 2g K_2HPO_4; 0.2g NaCl;; 0.2g $MgSO_4$; 0.015g $CaCl_2$, trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with pH 6.0 phosphate buffer (0.2M)) containing 2g/L vanillic acid which was incubated at 30°C with shaking at 200rpm for 24 hours. This starter culture was added to 5 litres of the same medium in a fermenter with the medium components made up to 1 litre with deionised water and vanillic acid added to a concentration of 1.5g L-$^{-1}$, immediately prior to inoculation. The fermenter contents were stirred at 30°C for 18 hours with the pH of the medium being maintained at pH 5.2 by the addition of 5M sodium hydroxide solution. After this time the medium pH was gradually reduced to 3.7 over 45 minutes by the

addition of 5M HCl and temperature was maintained at 30°C. Dissolved oxygen concentration was maintained at 70% of saturation throughout the process. Assay was by hplc as described above. Prior to the pH being allowed to drop at the 18 hour stage, the amount of vanillic acid in the system was measured at 1.48 g L^{-1}; vanillin was detected in solution at 0.02 g L-1 . As the reaction proceeded various amounts of vanillic acid or glucose were added as follows: 19.25 hours, 50g glucose; 20.25 hours, 3g vanillic acid; 21.5 hours, 4g vanillic acid; 23 hours, 4g vanillic acid. Substrate and product concentrations were measured as being: 19.25 hours, vanillic acid 1.5 g L^{-1}, vanillin 0.1 g L^{-1};: 20.25 hours, vanillic acid 1.16 g L^{-1}, vanillin 0.36 g L^{-1};: 21.5 hours, vanillic acid 1.07 g L^{-1}, vanillin 0.74 g L^{-1};: 23 hours, vanillic acid 1.06 g L^{-1}, vanillin 1.22 g L^{-1};: 26 hours, vanillic acid 0.83 g L^{-1}, vanillin 1.96 g L^{-1}.

Example 5: production of vanillin by A.fumigatus

A spore suspension of Aspergillus fumigatus (Zyl 747) was used to inoculate 5 litres of a minimal medium (ingredients described in Example 4(a), except for the addition of 3g L^{-1} vanillic acid rather than 1.5g L^{-1}). The fermenter contents were stirred without pH control at

30°C with the dissolved oxygen concentration being maintained at 60% of saturation. Assay was by hplc as described above. Substrate and product concentrations in the culture broth were as follows: 16 hours, vanillin 0.015g L^{-1}; 24 hours, vanillin 0.075g L^{-1}; 40 hours, vanillin 0.69g L^{-1}; 47 hours, vanillin 0.9 lg L^{-1}; 48.5 hours, 0.98g L^{-1}; 53.5 hours 1.02gL^{-1}; 112 hours, vanillic acid 1.32gL^{-1}, vanillin 1.09gL^{-1}

D) Preparations involving coniferyl alcohol (7)

Coniferyl alcohol is available from a number of natural and synthetic sources. In addition we have developed an efficient microbiological route from ferulic acid (1a).

Example 6: coniferyl alcohol (7) from ferulic acid (1a)

In a 1 litre conical flask, 200mL of presterilised yeast malt medium (4g glucose; 4g yeast extract; 10g malt extract; made up to 1 litre with deionised water) was inoculated (2% inoculum) with Rhizopus arrhizus (Zyl 779) (from a 24 hour seed flask of the same medium). Ferulic acid (400mg) was added, giving a final concentration of 2g/L substrate. This flask was incubated, with shaking at 200rpm, at 30°C and assayed by hplc. After 46 hours the culture broth was adjusted to pH7 and extracted twice with equal volumes of ethyl acetate. The evaporated solvent gave coniferyl alcohol as a yellow oil (150mg)

which solidified upon standing.

We have found that coniferyl alcohol (7) can be efficiently converted into coniferyl aldehyde (6) by means of Brevundimonas vesicularis.

Example 7: coniferyl aldehyde from coniferyl alcohol

To a pre-sterilised 250mL conical flask containing 50 mL of nutrient broth (No. 2) was added an inoculum of Zyl 295 (Brevundimonas vesicularis) from an agar plate culture. The organism was grown at 30°C for 24 hours with shaking at 200rpm. After this time coniferyl alcohol (50mg) was added to the flask to give a final concentration of substrate of 1 mg/mL. The flask was shaken as before and after 4 hours assay by HPLC showed that no substrate remained whilst there was a concentration of 0.82mg/mL of coniferaldehyde. This represents a 83% conversion of coniferyl alcohol to coniferaldehyde.

E) In situ product removal ("ISPR")

The principle involves carrying out a biotransformation in an aqueous phase which is in contact with, or is contacted with, an immiscible phase into which a product can pass (exclusively or preferentially). Possible advantages include a) the protection of a product from further reaction in the aqueous phase; b) avoidance of inhibition of product formation by a

microorganism by high product concentrations; c) enabling equilibrium reactions to convert a larger proportion of starting material into product; and d) ease of isolation of products. Furthermore ISPR can assist in the development of continuous systems.

ISPR is readily applied to systems where a polar substrate (e.g. a carboxylic acid such as ferulic or vanillic acid) is converted into a less polar product, which will be preferentially extracted by a nonpolar solvent such as a plant oil material, preferably food grade. Polar byproducts will also tend to stay in the aqueous phase.

Examples 8 and 9 correspond to Examples 4 and 5 but make use of ISPR.

Depending on the system, the immiscible phase may contact the aqueous phase while biotransformation is proceeding therein, or it may contact portions of the aqueous phase which have been withdrawn (temporarily) from the bioreactor. The withdrawn phase may be treated (e.g. by adjustment of pH) to facilitate extraction by the immiscible phase.

Example 8: production of vanillin by M.isabellinus with ISPR

A 5 litre culture of Micromucor isabellinus (Zyl 849) was grown in a fermenter as described in Example 4b.

The fermenter contents were stirred at 30°C for 20 hours with the pH of the medium maintained at 5.2 by the addition of 5M sodium hydroxide solution. After this time the medium pH was gradually reduced to 3.8 over 45 minutes by the addition of 5M HCl and was maintained at this pH thereafter; temperature was maintained at 30°C. Dissolved oxygen concentration was maintained at 70% of saturation throughout the process. Assay was by hplc as described above. The progress of the reaction was monitored until the concentration of vanillin in the culture broth reached 0.96 g L^{-1} . Throughout this period vanillic acid was added to the medium to maintain a concentration of 1.5 g L^{-1}. At this time an external vanillin extraction system was activated as follows: culture medium was continuously pumped from the fermenter through a filtration device such that the biomass was retained within the fermenter; culture medium exiting the fermenter was heated to 60°C and adjusted to pH 6.5 by the addition of 10M sodium hydroxide solution; this medium was fed into an extraction vessel containing 51itres of sunflower oil; the aqueous phase (maintained at 1 litre volume) and the oil phase were stirred vigorously by the use of an overhead stirrer to effect continuous selective extraction of vanillin from the culture medium into the oil phase; vanillic acid did not

extract into the oil and remained entirely in the aqueous phase; this aqueous phase was continually pumped back into the fermenter. Throughout the process, the volume in the fermenter and the volume in the extraction vessel remained relatively constant. The concentration of vanillic acid in the fermenter was maintained at approximately 1.5 g L^{-1} and the concentration of vanillin in the fermenter was maintained at a maximum of 1.5 g L^{-1}. Using this continuous external extraction facility the 5 litre culture of Micromucor isabellinus produced 18.9g vanillin over an operational period of approximately 20 hours. Vanillin can be recovered from sunflower oil by extraction into water, or alcohol, e.g. methanol or ethanol, or into an alcohol/water mixture. A suitable mixed solvent is 80% ethanol and 20% water. After evaporation of the extraction solvent, vanillin can be further purified by traditional techniques such as recrystallisation, sublimation etc.

Example 9: production of vanillin by A.fumigatus with ISPR

A sterile glass column (60mL volume) packed with a stainless steel support was filled with a minimal medium (ingredients described in Example 4) containing 2g L^{-1} vanillic acid and inoculated with spores of Aspergillus fumigatus (Zyl 747). Air was pumped into the base of the

column in order to aerate the system and effect efficient mixing. The column was allowed to stand at room temperature (22°C) for 70 hours. After this time substantial growth of the fungus had occurred and all had adhered to the stainless steel support material. The concentration of vanillin in solution was measured as $0.65gL^{-1}$. Maintaining aeration as described above, the contents of the column were continuously pumped out of the column into a separate extraction vessel containing 500mL sunflower oil and 200mL of the same minimal medium described previously. In addition, 1g vanillic acid was added to the aqueous phase. The contents of the extraction vessel were mixed thoroughly and the aqueous phase continuously pumped back through the column. Incubation of the column contents at approximately 30°C was achieved by jacketing the column with silicon tubing and pumping water through at a temperature of 34°C. The concentration of vanillin in both the oil and aqueous phase inside the extraction vessel was assayed (hplc, as described above) at intervals over an eight day period. Results, expressed as total vanillin yield from the system, were as follows: 24 hours, 113mg; 48 hours, 140mg; 72 hours, 269mg; 96 hours, 385mg; 168 hours, 597mg; 192 hours, 566mg.

The suitability of the system of example 9 for long-

term continuous use is demonstrated by Example 10.

Example 10: continuous production of vanillin using ISPR

A spore suspension of Aspergillus fumigatus (Zyl 747) was used to inoculate 3 litres of a minimal medium (ingredients described in Example 8 except for the addition of 3.3g L^{-1} vanillic acid rather than 1.5g) in a fermenter (5L working volume). The fermenter contents were stirred without pH control at 30°C with the dissolved oxygen concentration being maintained at 60% of saturation throughout the process. Assay was by hplc as described above. After 24 hours incubation, 0.15g L^{-1} vanillin was detected in solution. At this time 2 litres of sunflower oil containing 0.5g L^{-1} vanillic acid was added to the fermenter and incubation was continued as described previously. Over a period of 28 days, the oil phase in the fermenter was removed at frequent intervals and replaced with fresh oil containing 0.5g L^{-1} vanillic acid Vanillin production expressed as the total vanillin yield from the system was as follows:

Time (hours)	Vanillin yield (g)
48	0.64
72	1.43
96	2.71
168	4.60
192	5.12

216	5.58
240	6.20
264	6.87
336	9.16
360	10.32
384	11.50
408	12.25
432	12.80
504	13.80
575	14.50
665	16.26

After 665 hours the experiment was terminated; however, the fungus was still actively producing vanillin.

G) Conversion of caffeic acid into ferulic acid

Example 11

A nutrient agar plate culture of *Streptomyces griseus* was used as an inoculum for 50 mL of growth medium (5g soyabean flour; 20g glycerol; 5g yeast extract; 5g sodium chloride; 5g dipotassium hydrogen phosphate; made up to 1 litre with deionised water and the pH adjusted to 7.0) in a 250mL shake flask. This culture was incubated at 27°C with shaking at 220rpm for 63 hours prior to being used to inoculate (6mL) a similar flask containing the same growth medium. This flask was grown under the same conditions for 24 hours after which

caffeic acid (25mg) dissolved in dimethylformamide (DMF) (500μL) was added to give a final concentration of 0.5g/L caffeic acid and 1% DMF. Incubation resumed as before. Assay for caffeic acid and ferulic acid indicated a drop in caffeic acid concentration over 69 hours to 0.29g/L and an increase in ferulic acid content to 0.08g/L. Assay also indicated the co-production of isoferulic acid, in this type of reaction system, at a level approximately 10% that of ferulic acid production.

Example 12: the effect of glycerol on resting cell suspensions.

A culture of *Streptomyces* was grown as in example 18; that is, in a two stage seed and final growth procedure. After 24 hours growth in the second stage the cells were harvested by centrifugation, washed twice with pH 7.4 phosphate buffer, 0.1M, and 2g of wet cell mass (approximately 80mg dry cell weight) was measured into each of four flasks, each of which contained pH7.4 phosphate buffer, 0.1M (5mL), and 2.5mg of caffeic acid dissolved in DMF (1% final volume). The flasks were dosed with either 0%, 1%, 5% or 10% glycerol. In the absence of glycerol all of the caffeic acid had been eliminated from the system in 24 hours; with 1% glycerol all caffeic acid had gone in 60 hours; with 5% glycerol the loss of caffeic acid was slower again with detectable

quantities after 94 hours; with 10% glycerol there was very little loss of caffeic acid substrate. The presence of glycerol stabilises the substrate in the system. In the absence of glycerol the small amount of ferulic acid seen at 17 hours had been lost from the system at 24 hours; with 1% glycerol more ferulic acid was produced up to 60 hours but this had gone from the system by 94 hours; with 5% glycerol ferulic acid was seen to be produced and then partially eliminated from the system; with 10% glycerol the amount of ferulic acid seen rose over the 94 hours reaction, reaching a maximum concentration of 0.1g/L.

H) Use of Pseudomonas putida for vanillic acid production

Example 13 Conversion of ferulic acid to vanillic acid

A nutrient agar plate culture of *Pseudomonas putida* (zyl 503) was used a source of inoculum for 50 ml of growth medium (5 g ferulic acid; 20 g glucose; 2g KH_2PO_4; 5g $(NH_4)_2SO_4$; 0.2g NaCl; 0.22 g $MgSO_4$; 0.015g $CaCl_2$; 1 ml trace elements solution; 10 ml vitamins solution, made up to 1 litre with 0.2 M, pH 7.0 phosphate buffer) in a 250 ml conical shake flask. The culture was incubated at 30°C shaking at 250 rpm and assayed by hplc as described above. As the reaction proceeded, additional amounts of

ferulic acid were added as follows: 24.5 hours 0.25 g; 48 hours 0.125 g; 72 hours 0.05 g; 90 hours 0.25 g; 96 hours 0.5 g. Substrate and product concentrations were measured as being the following: 20.5 hours, ferulic acid 2.71 gl^{-1} ,vanillic acid 2.08 gl^{-1} ;24.5 hours, ferulic acid 1.51 gl^{-1}, vanillic acid 3.01 gl^{-1}; 48 hours, ferulic acid 2.4 gl^{-1} ,vanillic acid 7.0 gL^{-1}; 72 hours, ferulic acid 2.08 gl^{-1}, vanillic acid 9.5 gL^{-1}; 96 hours ferulic acid 4.47 gL^{-1}, vanillic acid 12.11g L^{-1}; 160 hours, ferulic acid 4.45 gL^{-1}, vanillic acid 19.05 gL^{-1}.

Example 14 Production of vanillic acid from maize fibre

To 30 g maize fibre in a 250 ml conical flask was added 100 ml of 8% w/v citric acid solution. The flask contents were heated at 85°C with efficient mixing for 16 hours. After this time, the stirred maize fibre suspension was neutralised by the dropwise addition of sodium hydroxide solution (10M). A hydrolytic enzyme preparation (500μl, Biofeed Plus, Novo Nordisk) was added to the suspension and the whole incubated at 45°C with continued mixing for 24 hours. Analysis was by hplc as described above. After 24 hours 3.3 gl^{-1} ferulic acid was detected in solution.

A culture of *Pseudomonas putida* (zyl 503) grown on

nutrient agar was used to inoculate a 250 ml shake flask containing 50 ml of minimal salts medium (4 g ferulic acid; 20 g glucose; 5 g $(NH_4)_2 SO_4$; 0.2 g NaCl; 2 g K_2HPO_4; 0.22 g $MgSO_4$; 0.015 g $CaCl_2$; 1 ml trace elements solution; 10 ml vitamins solution made up to 1 litre with pH 7.0 phosphate buffer 0.2 M. The flask contents were incubated at 30°C shaking at 250 rpm for 24 hours. After this time the culture was harvested by centrifugation (4000 x g, 20 minutes), washed once with 0.2 M pH 7.0 phosphate buffer to remove residual non maize derived ferulic or vanillic acid and finally resuspended in 2 ml of the same buffer (x 25 concentration). This concentrated cell suspension was added to a 20 ml aliquot of the hydrolysed maize suspension described above contained in a 100 ml conical flask. The concentration of ferulic acid in solution was measured as 2.8 gl^{-1} prior to incubation at 30°C with shaking at 250 rpm. The concentration of vanillic acid present throughout the incubation period was measured as being the following: 4 hours, 0.11 gl^{-1}; 24.5 hours, 1.44 gL^{-1}; 29.5 hours, 1.98 gL^{-1}; 31 hours, 2.0 gL^{-1}.

I) <u>Transformations of other substrates</u>

The conversion of ferulic acid into vanillic acid as in example 1 is the conversion of a cinnamic acid (AR-CH=CH-CO_2H) to a benzoic acid (AR-CO_2H). This can be

applied to other cinnamic acids, e.g. coumaric acid (4-hydroxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid).

Example 15: 4-hydroxybenzoic acid from coumaric acid

A seed stage culture of Rhodotorula glutinis (Zyl 702) was grown for 24 hours at 30°C with shaking at 200 rpm in a 250 mL shake flask containing 50 mL of a minimal medium (as defined in Example 1). This culture was used to inoculate (2%) a 250 mL shake flask containing 50 mL of the same minimal medium with the addition of 200 mg coumaric acid to give a final concentration of 4 gL^{-1}. Incubation conditions were as described previously and assay was by hplc as described above. Substrate and product concentrations were measured as the following: 18 hours, coumaric acid 1.75 gL^{-1}, 4-hydroxybenzoic acid 1.28g L^{-1}, 3,4 dihydroxybenzoic acid 0.27 gL^{-1}; 22 hours, coumaric acid 0.36 gL^{-1}, 4-hydroxybenzoic acid 1.92 gL^{-1}, 3,4-dihydroxybenzoic acid 0.44 gL^{-1}; 23 hours, coumaric acid 0.12 gL^{-1}, 4-hydroxybenzoic acid 2.10 gL^{-1}, 3,4-dihydroxybenzoic acid 0.47 gL^{-1}.

Example 16: protocatechuic acid (3,4-dihydroxybenzoic acid) from caffeic acid

To 400mL of sterilised yeast malt medium (4g glucose; 4g yeast extract; 10g malt extract; made up to 1 litre with deionised water) was added glucose (40g) and

caffeic acid (1g) and the whole was inoculated with spores of Paecilomyces variotti (Zyl 733) prior to incubation at 30°C with shaking at 200rpm. Further aliquots of glucose (20g) were added at 24 hours, 72 hours and 96 hours. After 168 hours, hplc assay indicated that there were 630 mg total of protocatechuic acid present in the reaction system, representing a 74% molar conversion. The culture broth was extracted with ethyl acetate (900mL) and assay showed that 527mg of protocatechuic acid had been recovered along with 45mg of unreacted caffeic acid. Evaporation of the dried solvent yielded 750mg of a pale yellow gum which was resuspended in diethyl ether (100mL) to give a red, granular, insoluble solid which was removed and the remaining solution evaporated to give 700mg of recovered solid which was 67% protocatechuic acid by assay and 6% caffeic acid. This solid was dissolved in diethyl ether (10mL) to which was then added a further 10mL of petroleum ether 46/60. Evaporation of this solution by blowing nitrogen over the solution gave a yellow oil from which the solution was decanted and evaporated to give a cream coloured solid (435mg) which was 96.3% protocatechuic acid by assay.

The conversion of vanillic acid to vanillin as in example 4 is the conversion of a hydroxybenzoic acid to a

hydroxybenzaldehyde. This can be applied to other benzoic acids, particularly hydroxybenzoic acids, e.g. 4-hydroxybenzoic acid (as produced in Example 11) or 3,4-dihydroxybenzoic acid (protocatechuic acid) as produced in Example 16).

Example 17: conversion of 4-hydroxybenzoic acid to 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol

A culture of Zygorhynchus moelleri (Zyl 851), grown on yeast malt agar, was used to inoculate a 250 mL conical flask containing 42 mL of culture medium (ingredients described in Example 8 with 20g L^{-1} (not 15g L^{-1}) glucose added) containing 100 mg 4-hydroxybenzoic acid. The culture broth was incubated at 30°C with shaking at 200 rpm. The progress of the reaction was assayed by hplc as described above. Substrate and product concentrations were measured as being the following: 24 hours, 4-hydroxybenzoic acid 2.26g L^{-1}, 4-hydroxybenzaldehyde trace amount, 4-hydroxybenzyl alcohol trace amount; 42 hours, 4-hydroxybenzoic acid 1.06g L^{-1}, 4-hydroxybenzaldehyde 0.53g L^{-1}, 4-hydroxybenzyl alcohol 0.55g L^{-1}; 66 hours, 4-hydroxybenzoic acid 0.1 g L^{-1}, 4-hydroxybenzaldehyde trace amount, 4-hydroxybenzyl alcohol 2.6g L^{-1}.

Example 18; conversion of 4-hydroxybenzoic acid to 4-hydroxybenzaldehyde

A culture of Trichoderma koningii (Zyl 751) grown on yeast malt agar, was used to inoculate 50 mL of a minimal medium (as defined in Example 4a) in a 250 mL conical flask. Prior to inoculation, 150mg (3 gL^{-1}) 4-hydroxybenzoic acid was added to the medium followed by incubation of the whole at 30°C with shaking at 200 rpm. Assay was by hplc and tlc as described above. After approximately 30 hours incubation, hplc analysis detected 0.3 gL^{-1} 4-hydroxybenzaldehyde in solution. This observation was further supported by tlc analysis which revealed the presence of material at R_f 0.59 consistent with a reference sample of 4-hydroxybenzaldehyde. The observed product also gave a positive colour reaction with dinitrophenylhydrazine solution.

Example 19: conversion of 3,4-dihydroxybenzoic acid to 3,4-dihydroxybenzaldehyde

A culture of Zygorhynchus moelleri (Zyl 851), grown on yeast malt agar, was used in inoculate a 250 mL conical flask containing 50 mL of culture medium (ingredients described in Example 8 with 20g L^{-1} (not 15g L^{-1}) glucose added) containing 100mg vanillic acid. The culture broth was incubated at 30°C with shaking at 200 rpm. The progress of the reaction was assayed by hplc as described above. After 42 hours incubation, approximately 50% of the vanillic acid had been converted

to vanillyl alcohol. At this time, 100mg of 3,4-dihydroxybenzoic acid (e.g. from Example 12, or extracted from onion skins) was added to the culture and incubation continued. After a further 6 hours incubation a new product corresponding to 3,4-dihydroxybenzaldehyde was detected at a concentration of approximately 0.20g L^{-1}. After 24 hours this product concentration had increased slightly to approximately 0.025g L^{-1}. After 48 hours the 3,4-dihydroxybenzaldehyde had been lost from the solution.

Example 20; Conversion of benzoic acid to benzaldehyde

In a 1 litre flask, 200 mL of medium, (50g glucose; 5g $(NH_4)_2SO_4)_4$;2g K_2HPO_4;0.2g NaCl;0.22g $MgSO_4$; 0.015g $CaCl_2$ 1ml trace element solution, 1mL; 10ml vitamins solution; made up to 1 litre with deionised water), was inoculated with a 10μL loopful of spores of Trichoderma koningii (Zyl 751) after the addition of 50mg of benzoic acid. This was incubated at 30°C with shaking at 200rpm. By hplc analysis, benzaldehyde was first detected in the culture broth at 25 hours incubation. Over the next 5 hours benzaldehyde concentration rose to 0.165g/L; at this time a concentration of 0.1g benzyl alcohol was also present in the solution.

NB the yields of the aldehydes in examples 17 - 20 could doubtless be improved by the use of ISPR.

The conversion of vanillyl alcohol into the aldehyde in example 3 can also be applied to other benzyl alcohols, e.g. 4-hydroxybenzyl alcohol as produced in example 17.

Example 21: conversion of 4-hydroxybenzyl alcohol to 4-hydroxybenzaldehyde

The methodology described in Example 19 was followed but at the 24 hour stage of growth of the organism 4-hydroxybenzyl alcohol was added to the flask to give a final concentration of 1 mg/mL. Monitoring the progress of the reaction by hplc showed that the amount of substrate dropped to 10% of the original after a further 28 hours at which stage 4-hydroxybenzaldehyde had reached a concentration of 0.37 mg/mL.

The conversion of caffeic acid into ferulic acid (e.g. Example 11) is the selective methylation of a meta-hydroxy group in preference to a para-hydroxy group. This can be applied to other polyhydroxy aromatics, e.g. protocatechuic acid (3,4,dihydroxybenzoic acid).

Example 22: conversion of protocatechuic acid into vanillic acid

S.griseus NRRL 8070 was grown from spores on a 50 ml scale in a medium containing 20g/L glucose and tryptone soy broth (30g/L).

After 72hr growth this culture was used as a 10% innoculum for 200ml scale bioconversion in 1L flasks. These were grown for 24hrs before substrate was added in the form of a 200mg protocatechuic acid/ml DMF solution. After 72hr a 10% conversion of PCA into vanillic acid and isovanillic acid in a 5.6:1 ratio was obtained.

J) Selective screening for organisms producing aldehydes from carboxylic acids

i) Preparation of agar plates

Minimal salts agar: 20g glucose; 5g $(NH_4)_2SO_4$; 2g K_2HPO_4; 0.2g NaCl; 0.22g $MgSO_4$; 0.015g $CaCl_2$; 10ml trace element solution; 1ml vitamins solution; 20g agar and 2g vanillic or ferulic acid made up to 1 litre with either deionised water (fungal isolation) or with 0.2M, pH 7.0, sodium phosphate buffer (bacterial isolation).

Filter paper (Whatman No.1) was cut into discs with a diameter of 90 mm and sterilised by autoclaving. Single discs were placed into 90mm sterile petri dishes prior to the pouring of a minimal salts agar described above.

ii) Preparation of soil samples

To 2ml deionised water was added approximately 100mg soil. The resulting suspension was mixed thoroughly

(vortex mixer); allowed to stand at room temperature (22°C) for 1 hour followed by further mixing to distribute suspended material. The macroscopic solids were allowed to settle for approximately 10 minutes and the supernatant (100μl) applied to the prepared minimal salts agar plates using a spread plate technique. Plates were incubated at 28°C until colony development was observed (approx. 5 days)

iii) Selective visualisation of aldehyde producing strains

In order to visualise vanillic acid or ferulic acid biotransformation products the following procedure was followed:

Agar was lifted from the base of each petri dish by inserting a spatula beneath the filter paper disc, followed by the injection of 1ml of dinitrophenyl hydrazine (DNP) solution (0.4% DNP in 2M HCl). The agar was replaced in the dish and the DNP solution allowed to permeate through the agar. Colonies producing aldehyde products were visualised by the presence of an orange/red zone surrounding the colony against a pale yellow background. Colonies producing alcohol products were visualised by the presence of a dark yellow zone surrounding the colony, against the pale yellow

background.

Newburn [illegible]
PG collected
24/2/03